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(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).		Published Without international search report and to be republished upon receipt of that report.	
(72) Inventors: CAHOON, Edgar, B.; Building 463, Brookhaven National Laboratories, Upton, NY 11973 (US). OHLROGEE, John, B.; 2123 Lagoon Drive, Okemos, MI 48864 (US).			
(74) Agents: YATES, Michael, E. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).			

**(54) Title:** METHODS AND COMPOSITIONS RELATING TO PLANT  $\Delta^6$  PALMITOYL-ACYL CARRIER PROTEIN DESATURASE

**(57) Abstract**

A plant  $\Delta^6$  palmitoyl-acyl carrier protein desaturase, the gene encoding the desaturase, and transgenic plants and plant cells containing the heterologous DNA encoding the desaturase are described. The desaturase introduces a double bond at the sixth carbon atom from the carboxyl end of a 16 carbon saturated fatty acid, and is therefore useful in production of plant seeds having a modified fatty acid profile.

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## METHODS AND COMPOSITIONS RELATING TO PLANT $\Delta^6$ PALMITOYL-ACYL CARRIER PROTEIN DESATURASE

### Field of the Invention

The present invention relates to plant fatty acid desaturases. More particularly, the present invention relates to plant  $\Delta^6$  palmitoyl-acyl carrier protein desaturases, the genes encoding such desaturases, transgenic plants and plant cells containing DNA encoding such desaturases, and methods for altering the fatty acid profile of plant seeds through the use of such desaturases.

### BACKGROUND

Fatty acid desaturases of plants have received considerable attention because of their contributions to the physiology and economic value of plants. The activity of fatty acid desaturases, for example, may be a component of the ability of certain species to adjust levels of membrane unsaturation in response to stresses such as chilling (1-3). In addition, the degree of fatty acid unsaturation resulting from desaturase activity is often a major determinant of the nutritional and industrial quality of plant seed oils (4).

Plants typically contain a variety of fatty acid desaturases. The most numerous of these are membrane-associated desaturases that use fatty acids bound to glycerolipids as substrates (5). In addition, the synthesis of oleic acid ( $18:1\Delta^9$ ) in plants and certain other organisms such as *Euglena* (photoauxotrophic) is catalyzed by a desaturase that functions on fatty acids esterified to acyl carrier protein (ACP) (6-8). This enzyme, the  $\Delta^9$  stearoyl ( $18:0$ )-ACP desaturase (EC 1.14.99.6), displays soluble activity in contrast to all previously characterized desaturases (6). In the presence of radiolabeled  $18:0$ -ACP and cofactors including NADPH, ferredoxin (Fd), and ferredoxin-NADPH reductase, the activity of the  $\Delta^9$   $18:0$ -ACP desaturase is readily detectable in extracts of most plant tissues (9). Due in part to its soluble nature and relative ease of assay, the  $\Delta^9$   $18:0$ -ACP desaturase has been purified from several plant sources (10-14), and a number of cDNAs encoding

this enzyme have been isolated (12-20). In addition to the  $\Delta^9$ 18:0-ACP desaturase, a  $\Delta^4$ 16:0-ACP desaturase has recently been identified in plants (21, 22). This enzyme is a component of the petroselinic acid (18:1 $\Delta^6$ ) biosynthetic pathway in endosperm of coriander (*Coriandrum sativum* L.) and other Umbelliferae species. Translation of a cDNA for the  $\Delta^4$ 16:0-ACP desaturase has revealed that this enzyme shares extensive amino acid identity with the  $\Delta^9$ 18:0-ACP desaturase (21).

The existence of structurally related acyl-ACP desaturases with different substrate recognition and double bond-positioning properties offers the opportunity to compare the active site structures of members of this family of enzymes using techniques such as site-directed mutagenesis and x-ray crystallography. Information gained from this research could potentially lead to the design of desaturases capable of producing new industrially useful isomers of monounsaturated fatty acids. These studies would be aided by the isolation of cDNAs for other variant acyl-ACP desaturases in addition to those for the  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases. A potential source of such a desaturase is seed of *Thunbergia alata* (Acanthaceae family). The oil of this tissue consists of more than 80% weight of the unusual fatty acid  $\Delta^6$  hexadecenoic acid (16:1 $\Delta^6$ ) (23). We have used biochemical and molecular biological approaches to examine whether 16:1 $\Delta^6$  is synthesized by the activity of a unique acyl-ACP desaturase that is related to the  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases.

#### **SUMMARY**

The present invention therefore relates to an isolated  $\Delta^6$  palmitoyl acyl carrier protein desaturase and DNA encoding same, transgenic plants and plant cells containing heterologous DNA encoding said desaturase, mRNA derived from DNA encoding such a desaturase, and vectors containing DNA encoding the desaturase. The present invention also relates to a method for modifying the fatty acid content of a plant seed involving the introduction of a double bond at the sixth carbon atom from the carboxyl end of the saturated 16 carbon fatty acid, for example palmitic acid. Additionally, the present

invention relates to plant seeds having a modified fatty acid content derived through the method of the invention.

An objective of the invention is to provide a mechanism for the modification of fatty acids in order to enhance the industrial and nutritional quality of plant seed oils. As part of this invention, we have developed a method for introducing a double bond at a novel position in a saturated fatty acid. The invention entails the identification of the enzyme (a  $\Delta^6$ -palmitoyl-acyl carrier protein desaturase) involved in the biosynthesis of the unusual fatty acid  $\Delta^6$ -hexadecenoic acid. In addition, a complementary DNA (cDNA) was isolated for this desaturase. Expression of the cDNA in *Escherichia coli* resulted in the production of a catalytically active  $\Delta^6$ -palmitoyl-acyl carrier protein desaturase.

This invention offers the opportunity to synthesize a monounsaturated fatty acid with a double bond positioned at the sixth carbon atom from the carboxyl end of the fatty acid molecule. The invention also allows for unsaturation to be introduced into a saturated 16 carbon fatty acid (palmitic acid). These two features of the invention allow for the production of a monounsaturated fatty acid that is not normally found in conventional plant seed oils. The major monounsaturated fatty acid present in seed oils is oleic acid, an 18 carbon fatty acid with a double bond positioned at the ninth carbon atom from the carboxyl end of the molecule.

The ability of the invention to introduce a double bond into palmitic acid offers the potential for reducing the saturated fatty acid content of vegetable oils. Typically, the presence of the 16 carbon saturated fatty acid palmitic acid limits the nutritional quality of seed oils. Therefore, the invention may allow for the production of a seed oil with reduced palmitic acid content. As a result, such an oil would presumably be less harmful to human health.

In addition, the product of the invention,  $\Delta^6$ -hexadecenoic acid, may be useful as a chemical precursor of certain industrial feedstocks. Because the double bond of  $\Delta^6$ -hexadecenoic acid is located at the sixth carbon atom, this fatty acid can be oxidatively cleaved to form adipic acid (a six carbon

dicarboxylic acid) and decanoic acid (a ten carbon fatty acid). Adipic acid is a precursor of nylon 66 and is currently derived from petroleum by-products.

Furthermore, the use of  $\Delta^6$ -hexadecenoic acid has been proposed as an emollient in cosmetics (U.S. Patent No. 4,036,991).

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**FIGURE 1** is an autoradiogram of intact products of [ $^{14}\text{C}$ ]16:0-ACP desaturase assays conducted with a 100,000 x g supernatant of *T. alata* endosperm extracts (16:1), and the methyl ester of 16:0 derived from unreacted [ $1\text{-}^{14}\text{C}$ ]16:0-ACP(16:0). The Std. lane contains methyl [ $1\text{-}^{14}\text{C}$ ]16:0 (16:0), [ $1\text{-}^{14}\text{C}$ ]18:1 $\Delta^9$ ( $\Delta^9$ ), and [ $1\text{-}^{14}\text{C}$ ]18:1 $\Delta^6$ ( $\Delta^6$ ).

**FIGURE 2** is an autoradiogram of oxidized products of [ $^{14}\text{C}$ ]16:0-ACP desaturase assays. Shown are the permanganate-periodate oxidation products of the methyl ester derivatives of 16:1 formed by acyl-ACP desaturation activity in *T. alata* endosperm homogenates. [ $\text{U-}^{14}\text{C}$ ]16:0-ACP was used as the substrate for this assay.

**FIGURE 3** shows the time course of [ $1\text{-}^{14}\text{C}$ ]16:0-ACP desaturase activity in a 100,000 x g supernatant of a *T. alata* endosperm homogenate. Assays were conducted with 118 pmol of [ $1\text{-}^{14}\text{C}$ ]16:0-ACP and 23  $\mu\text{g}$  of protein.

**FIGURE 4** shows the effect of catalase, ferredoxin (Fd), nitrogen ( $\text{N}_2$ ), potassium cyanide (KCN), or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) on  $\Delta^6$ 16:0-ACP desaturase activity in *T. alata* endosperm extracts. Assays were conducted for 10 min using 118 pmol of [ $1\text{-}^{14}\text{C}$ ]16:0-ACP and 23  $\mu\text{g}$  of protein from a 100,000 x g supernatant of endosperm homogenate. Assays with potassium cyanide and hydrogen peroxide contained 1mM of each compound, and catalase was omitted from assays containing hydrogen peroxide. (n.d.—not detected).

**FIGURE 5** is the nucleotide sequence of the cDNA insert of pTAD4 ( $\Delta^6$ ) and a comparison of the deduced amino acid sequences of pTAD4 ( $\Delta^6$ ) and cDNAs for the coriander  $\Delta^4$ 16:0-ACP desaturase ( $\Delta^4$ ) (see Ref. 21) and the castor  $\Delta^9$ 18:0-ACP desaturase ( $\Delta^9$ ) (see Ref. 13). Identical amino acids are indicated by colons. Amino acids that are absent relative to the castor  $\Delta^9$ 18:0-ACP desaturase are indicated by dashed lines. Alignment of the

nucleotide sequence of the cDNA insert of pTAD4 is maintained with a dotted line. The underlined alanine at amino acid 33 is the likely start of the native peptide encoded by pTAD4.

**FIGURE 6** compares the  $\Delta^6$ 16:0-ACP desaturase activity of extracts of *E. coli* BL21 pLysS containing only the vector pET3d or pET3d with insert derived from the *T. alata* cDNA of pTAD4 (with or without isopropyl-1-thio- $\beta$ -D-galactopyranoside induction). The methyl ester of 16:1 formed by the *E. coli*-expressed desaturase was separated from methyl 16:0 of the unreacted substrate by argentation TLC as shown. Assays were conducted for 60 min using 230  $\mu$ g of *E. coli* protein and 118 pmol of [1- $^{14}$ C]16:0-ACP. Shown in the standard chromatogram are (std.) are methyl [ $^{14}$ C]16:0, -18:1 $\Delta^9$ , and -18:1 $\Delta^6$ .

**FIGURE 7** shows substrate specificity and ferredoxin dependence of the *E. coli*-expressed *T. alata*  $\Delta^6$ 16:0-ACP desaturase encoded by the cDNA insert of pTAD4. Assays were performed for 10 minutes using 118 pmol of either [1- $^{14}$ C]14:0-, 16:0-( $\pm$  ferredoxin, Fd), or 18:0-ACP and 65  $\mu$ g of total *E. coli* protein.

**FIGURE 8** shows a mass spectrum of derivatives of [1- $^{14}$ C]16:1 formed by 16:0-ACP desaturase activity in extracts of *E. coli* expressing the mature peptide-coding region of pTAD4. The [1- $^{14}$ C]16:1 desaturation product was converted to a methyl ester derivative and reacted with dimethyl sulfoxide prior to mass spectral analysis.

### **DETAILED DESCRIPTION**

#### **Experimental Procedures**

**Plant Material** - Studies were conducted using developing endosperm dissected from fruits of *T. alata* Bojer ex Sims (black-eyed susan vine) (Northrup King, Minneapolis, MN). Fruits were collected from plants grown either outdoors in pots during summers in East Lansing, Michigan or under greenhouse conditions with natural illumination. In the latter case, flowers required hand pollination for adequate fruit set. Endosperm was frozen in liquid nitrogen following dissection and stored at -70°C until use in enzyme assays or RNA extraction.

**Acyl-ACP D saturation Assays** - Approximately 200-300 mg of developing *T. alata* endosperm was homogenized in 3 ml of buffer consisting of 100 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 1 mM isoascorbate, 10% (v/v) glycerol, and 1.5% (w/v) polyvinylpolypyrrolidone using an Elvehjem tissue grinder. Debris and polyvinylpolypyrrolidone were subsequently removed by centrifugation at 14,000 x g for 5 min. The supernatant was then passed through two layers of miracloth (Calbiochem) and spun for an additional 10 min at 30,000 x g. The soluble phase was removed while attempting to avoid recovery of the floating fat layer. A portion of contaminating fat was extracted by passing the supernatant through glass wool loosely packed in a Pasteur pipette. The supernatant from the 30,000 x g spin was further clarified by centrifugation at 100,000 x g for 60 min. All centrifugation steps were performed at 5°C. The resulting supernatant was used immediately for desaturation assays described below or frozen in aliquots in liquid N<sub>2</sub> and stored at -70°C until further use. Of note, extracts developed a brown color, presumably due to extensive phenolic oxidation, when maintained at -20°C for longer than 1-2 weeks.

Acyl-ACP desaturation assays were based on those previously described (8, 9). Assays were performed in a total volume of 150 µl in loosely capped 13 x 100-mm glass tubes and consisted of 1.25 mM NADPH (from a freshly prepared stock in 100 mM Tricine, pH 8.2), 3.3 mM ascorbate, 0.7 mM dithiothreitol, 8000 units of bovine liver catalase (Sigma), 5 µg of bovine serum albumin (Fraction V) (Sigma), 20 µg of spinach ferredoxin (Sigma), 80 milliunits of spinach ferredoxin:NADPH reductase (Sigma), 33 mM PIPES, pH 6.0, and 118 pmol of [1-<sup>14</sup>C] acyl-ACP or -CoA. Reactions were started with the addition of the 100,000 x g supernatant of homogenized *T. alata* endosperm (typically 20-25 µg of total protein) and were conducted at room temperature (-22°C) with shaking (100 revolutions/min). Assays were terminated with the addition of 850 µl of 2.35 M NaOH and carrier fatty acids (30 µg of palmitic and petros linic acid). The stopped reactions were then heated at 85°C for 1 hour. Following acidification with 350 µl of 4M H<sub>2</sub>SO<sub>4</sub>, the resulting free fatty acids were recovered by three extractions with 2.5 ml



of hexane. Fatty acids were converted to methyl ester derivatives with 10% (w/v) boron trichloride in methanol (Alltech) using the method described by Morrison and Smith (24). Reaction products were then analyzed on 15% AgNO<sub>3</sub> TLC plates developed sequentially to heights of 10 and 20 cm in toluene at -20°C. TLC plates were prepared as described previously (25). Radioactivity was detected by autoradiography and quantified by liquid scintillation counting of TLC scrapings in a non-aqueous complete mixture.

To confirm the identity of 16:1 $\Delta^6$  produced from palmitoyl-ACP, assays were conducted as described above using [U-<sup>14</sup>C] palmitoyl-ACP as the substrate. The methyl ester derivative of the monounsaturated product was purified by argentation TLC as described above and eluted from TLC scrapings with hexane/ethyl ether (2:1, v/v). The monounsaturated methyl ester was then cleaved at its double bond using permanganate-periodate oxidation (26). Chain lengths of oxidation products were determined relative to [<sup>14</sup>C] fatty acid standards by reverse-phase TLC using a mobile phase of acetonitrile/methanol/water (75:25:0.5).

Inhibition of desaturase activity was examined by supplementing assays with 1 mM KCN (neutralized) or 1 mM H<sub>2</sub>O<sub>2</sub>. In the latter case, catalase was omitted from reactions. Oxygen dependence of desaturase activity was characterized by purging assay tubes completely with nitrogen prior to and after addition of plant extract, and the reaction tube was tightly capped for the duration of the assay.

Radiolabeled acyl-ACPs were synthesized enzymatically using *Escherichia coli* ACP according to the method of Rock and Garwin (27). The following fatty acids were used in the synthesis of acyl-ACPs: [1-<sup>14</sup>C] myristic acid (American Radiolabeled Chemicals, St. Louis, MO) (specific activity 55 mCi/mmol), [1-<sup>14</sup>C] palmitic acid (NEN Dupont) (specific activity 58 mCi/mmol), [U-<sup>14</sup>C] palmitic acid (NEN Dupont) (specific activity 800 mCi/mmol), and [1-<sup>14</sup>C] stearic acid (American Radiolabeled Chemicals) (specific activity 55 mCi/mmol). [1-<sup>14</sup>C] Palmitoyl-CoA (specific activity 52 mCi/mmol) was purchased from Amersham Corp. A [<sup>14</sup>C] p-troselinic acid

standard was prepared by incubation of coriander endosperm slices in [1-<sup>14</sup>C] acetate as described previously (25).

***T. alata* Endosperm cDNA Library Construction** - Total RNA was isolated from *T. alata* endosperm using the method of Hall et al. (28). RNA was then passed through a cellulose (Sigma Cell 50, Sigma) column in order to reduce amounts of polysaccharides potentially recovered along with the RNA.

Poly(A)<sup>+</sup> RNA was enriched by passing total RNA once through a column of oligo (dT) cellulose (Pharmacia LKB Biotechnology Inc.) and subsequently used in the construction of a Uni-ZAP XR (Stratagene) cDNA expression library according to the instructions of the manufacturer. A portion of the total amplified library packaged in phage was mass excised (29) yielding pBluescript II SK(-) harboring cDNA inserts. The recovered plasmid DNA was used for cDNA isolation by colony hybridization and polymerase chain reaction (PCR) amplification as described below.

**PCR Amplification of Nucleotide Sequences Encoding Acyl-ACP Desaturases** - Fully degenerate sense and antisense oligonucleotides were prepared that corresponded respectively to the conserved amino acid sequences Gly-Asp-Met-Ile-Thr-Glu-Glu and Glu-Lys-Thr-Ile-Gln-Tyr-Leu present in  $\Delta^9$ 18:0 - (13, 15-20) and  $\Delta^4$ 16:0-ACP desaturases (21). The sequence of the resulting sense and antisense oligonucleotides were 5'-GG(A/C/G/T)GA(C/T)ATGAT(A/C/T)AC(A/C/G/T)GA(A/G)GA-3' and 5'-A(A/G)(A/G)TATTG(A/G/T)AT(A/C/G/T)GT(C/T)TT(C/T)TC-3', respectively. Included on the 5' terminus of each oligonucleotide was sequence (5'-CAUCAUCAUCAU-3' or 5'-CUACUACUACUA-3') that allowed for insertion of PCR products into the pAMP1 vector (Life Technologies, Inc.). Template for PCR amplification was generated by transformation of the SOLR strain (Stratagene) of *E. coli* with an aliquot of the mass-excised *T. alata* endosperm cDNA library. Following growth of transformed *E. coli* to stationary phase in 3 ml of liquid culture, plasmid DNA was purified for use as template in PCR amplification. Reactions were performed in a 50- $\mu$ l volume and consisted of 10  $\mu$ M sense and antisense oligonucleotides, 150-300 ng of plasmid DNA

derived from the *T. alata* cDNA library. 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1 x *Taq* reaction buffer (Life Technologies, Inc.), and 5 units of *Taq* polymerase (Life Technologies, Inc.). Temperature conditions for PCR amplification were 5 min at 95°C and 25 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C. This was followed by an additional 10 min extension at 72°C. PCR fragments of approximately 215 base pairs were gel-purified, ligated into the pAMP1 vector using the CloneAmp system (Life Technologies, Inc.) according to the manufacturer's protocol, and introduced into *E. coli* DH5a (Life Technologies, Inc.). The resulting colonies were screened using colony hybridization as described by Sambrook et al. (30). A "negative" screening protocol was used to reduce the chances of reisolating cDNAs (pTAD1, 2, and 3) encoding  $\Delta^9$ 18:0-ACP desaturases that were previously obtained by antibody screening of the *T. alata* endosperm cDNA library (19). DNA probes for library screening were formed by PCR amplification of portions of pTAD 1,2, and 3. Primers and PCR reaction conditions were the same as those described above. An equimolar mixture of the PCR products derived from pTAD1, 2, and 3 was used as template for the synthesis of [ $\alpha$ -<sup>32</sup>P]dCTP random-primed labeled probes. Hybridization of plasmids of lysed colonies with radiolabeled probes was carried out in 6 x SSC and 0.25% (w/v) non-fat dry milk with shaking for 4 hours at 53°C as described by Sambrook et al. (30). Filters were washed three times in 1 x SSC and 0.1% SDS at 60°C (45 min/wash) and exposed to autoradiography. Plasmid DNA was subsequently isolated from 10 colonies which displayed little or no hybridization to the probes. Nucleotide sequence of the inserts of these plasmids was obtained by dideoxy chain termination using Sequenase 2.0 (United States Biochemical Inc.) according to the manufacturer's instructions. Two classes of plasmids were identified (designated pEC6 and 7), both of which contained inserts encoding portions of apparent acyl-ACP desaturases (based on amino acid identity with known  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases).

**Screening of a *T. alata* Endosperm cDNA Library for a Full-length Divergent Acyl-ACP Desaturase** - Aliquots of the mass excised *T. alata* endosperm cDNA library were used to transform *E. coli* SOLR cells.

Approximately 50,000 colonies were screened using colony hybridization as described previously (30). Nucleotide probes for screening were generated by [ $\alpha$ - $^{32}$ P]dCTP random-primed hexamer labeling of inserts of pEC6 and 7. Hybridization and washing conditions were the same as those described above. Colonies containing plasmid DNA that strongly hybridized to the probe derived from pEC6 were isolated, and nucleotide sequence was obtained from both strands of the longest cDNA insert (the corresponding plasmid was designated pTAD4) using Sequenase 2.0. Because of a relative lack of abundance, colonies containing plasmid hybridizing to the pEC7-derived probe were not further characterized.

***E. coli* Expression of a Putative cDNA for  $\Delta^6$  Palmitoyl-ACP Desaturase** - To determine the activity of the desaturase encoded by pTAD4, the portion of the clone corresponding to the mature peptide (total protein minus plastid transit peptide) was expressed in *E. coli*. This region of the cDNA insert of pTAD4 was first amplified by PCR using Vent DNA polymerase (New England Biolabs). The nucleotide sequence of the sense primer was 5'-GCTTCGACTATTACTCAC3-. M-13(-20) forward primer was used as the antisense oligonucleotide. The PCR product was blunt-end ligated into the *Nco*I site of the *E. coli* expression vector pET3d (Novagen) as described (30). The *Nco*I-digested vector had been previously treated with the Klenow fragment of DNA polymerase I to fill-in 5' protruding ends. The junction between the vector and the 5' terminus of the insert was sequenced to confirm that the PCR product was ligated into pET3d in the proper reading frame. This construct was subsequently introduced into the *E. coli* strain BL21 pLysS and grown in LB media with carbenicillin (125  $\mu$ g/ml) and chloroamphenicol (30  $\mu$ g/ml) selection. At a cell density of OD<sub>600</sub> ~ 0.8, cultures were induced with the addition of isopropyl-1-thio- $\beta$ -D-galactopyranoside to a final concentration of 0.5 mM and grown for an additional 4 hours. Cells were then washed in 50 mM Tris-HCl, pH 7.5, lysed by two freeze-thaw cycles (using a liquid nitrogen bath for freezing and a 22°C water bath for thawing). Lysates were then incubated with bovine pancreas DNase I (Boehringer Mannheim) (20  $\mu$ g/ml) for 15 min at 22°C. The

extract was subsequently centrifuged at  $14,000 \times g$  for 5 min. The resulting supernatant was used for acyl-ACP desaturation assays as described above. Radiolabel in the TLC-analyzed reaction products was detected using a Bioscan System 200 image scanner. The double bond position of the monounsaturated product was determined by gas chromatography-mass spectrometry analysis of its dimethyl disulfide derivative (31). In these studies, the desaturation assays described above were scaled up 6-fold, and reactions were conducted with 2.6 nmol of [ $1\text{-}^{14}\text{C}$ ] 16:0-ACP and 1.1 mg of soluble protein of lysed *E. coli* expressing the *T. alata* cDNA. Assays were conducted for 4 hours. High protein concentrations and long incubation periods were used to ensure the synthesis of sufficient amounts of monounsaturated fatty acid for mass spectral analyses. Reaction products were converted to fatty acid methyl esters as described above and subsequently reacted with 100  $\mu\text{l}$  of an iodine solution (60 mg/ml ethyl ether) and 350  $\mu\text{l}$  of dimethyl disulfide (Aldrich). After 3 hours of incubation with shaking (250 revolutions/min) at  $37^\circ\text{C}$ , dimethyl disulfide derivatives of unsaturated fatty acid methyl esters were extracted as described previously (32). These derivatives (dissolved in hexane) were then analyzed by gas chromatography-mass spectrometry using a Hewlett Packard PH5890II gas chromatograph interfaced with a HP5971 mass selective detector. Separation of analytes was achieved using a DB23 (30 m x 0.25 mm inner diameter) column (J&W Scientific) with the oven temperature programmed from  $185^\circ\text{C}$  (3 min hold) to  $230^\circ\text{C}$  at rate of  $2.5^\circ\text{C}/\text{min}$ .

## **Results**

***Detection of a Soluble  $\Delta^6$  Palmitoyl-ACP Desaturase in T. alata Endosperm Extracts*** - The seed oil of *T. alata* is composed of nearly 85% weight of the unusual monounsaturated fatty acid  $\Delta^6$  hexadecenoic acid (16:1 $\Delta^6$ ). To examine the metabolic origin of the double bond of this fatty acid, the  $100,000 \times g$  supernatant of a homogenate of developing *T. alata* seed endosperm was incubated with [ $^{14}\text{C}$ ]16:0-ACP and potential desaturase cofactors. Using this assay system, substantial amounts of 16:0-ACP desaturase activity were detected in the soluble endosperm extract (Fig. 1).

In the absence of a radiolabeled standard for 16:1 $\Delta^6$ , two independent analytical methods indicated that the double bond of the resulting 16:1 moiety was positioned at the  $\Delta^6$  carbon atom: 1) the 16:1 desaturation product displayed mobility on argentation TLC plates similar to that of the  $\Delta^6$  monounsaturated fatty acid petroselinic acid (18:1 $\Delta^6$ ) when these molecules were analyzed as methyl ester derivatives (Fig. 1) and 2) permanganate-periodate oxidation of the methyl ester of the 16:1 desaturation product gave rise to a molecule with mobility on reverse-phase TLC equivalent to that of decanoic acid (10:0) (Fig. 2, *Product B*) as well as to an acyl moiety containing a lesser number of carbon atoms (Fig. 2, *Product A*).

**Substrate Properties of the  $\Delta^6$  Acyl-ACP Desaturase** - To confirm that the  $\Delta^6$  desaturase identified above is most active with 16:0-ACP, assays were conducted using  $^{14}\text{C}$ -saturated acyl-ACP substrates containing 14, 16, and 18 carbons. As with 16:0-ACP (described above),  $\Delta^6$  desaturase activity was also detected when [1- $^{14}\text{C}$ ]14:0- and 18:0-ACP were reacted with a 100,000 x *g* supernatant of a *T. alata* endosperm homogenate. Following derivitization, a portion of the desaturation products resulting from 18:0-ACP comigrated on argentation TLC with the methyl ester of petroselinic acid, which was resolvable in this system from methyl oleic acid (data now shown). Similarly, the desaturation product arising from 14:0-ACP migrated in the expected position for a fatty acid containing a  $\Delta^6$  double bond (data not shown). Therefore, it appears that the  $\Delta^6$  desaturase of *T. alata* endosperm positions the placement of unsaturation with regard to the carboxyl end of fatty acid substrates. This double bond positioning property has been previously observed with the  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases (22, 33).

Under the assay conditions used,  $\Delta^6$ 16:0-ACP desaturase activity in the 100,000 x *g* supernatant of a *T. alata* endosperm homogenate was essentially linear over 10 min (Fig. 3). When assays were conducted over this time period, the specific activity of the  $\Delta^6$  desaturase was approximately 7-fold higher using [1- $^{14}\text{C}$ ]16:0-ACP as a substrate rather than either [1- $^{14}\text{C}$ ]14:0- or 18:0-ACP (Table 1). Values obtained with the latter substrate, however, were obscured because of the presence of competing  $\Delta^9$ 18:0-ACP

desaturase activity in the endosperm extract. Finally, no desaturase activity was detected when [1-<sup>14</sup>C]16:0-CoA was presented as a potential substrate. Overall, these results indicate that the  $\Delta^6$  desaturase is most active *in vitro* with 16:0 esterified to ACP.

**TABLE I**

*In vitro* substrate specificities of acyl-ACP or -CoA desaturases of *T. alata* endosperm

Desaturase assays were conducted for 10 min using 118 pmol of [1-<sup>14</sup>C]acyl-ACP or -CoA substrate and 23  $\mu$ g of total protein from 100,000 x g supernatant of a *T. alata* endosperm homogenate.

<u>Substrate</u>	<u>Monounsaturated products<sup>a</sup></u>	
	$\Delta^6$	$\Delta^9$
	<i>pmol/min/mg protein</i>	
14:0-ACP	13	ND <sup>b</sup>
16:0-ACP	99	ND
16:0-CoA	ND	ND
18:0-ACP	12	173 <sup>c</sup>

<sup>a</sup>14:1 $\Delta^6$ , 18:1 $\Delta^6$ , and 18:1 $\Delta^9$  were identified by the mobilities of these fatty acids on argentation TLC plates.

<sup>b</sup>Not detected.

<sup>c</sup>Assay conditions were adjusted only for the linear measurement of  $\Delta^6$  desaturase activity. Therefore this value may underestimate the specific activity of  $\Delta^9$ 18:0-ACP desaturase.

***C factors and Inhibitors of  $\Delta^6$  Palmitoyl-ACP Desaturase Activity -***

Additional *in vitro* assays were conducted to compare the functional properties of the  $\Delta^6$ 16:0-ACP desaturase with those previously determined for the  $\Delta^9$ 18:0-ACP desaturase (7, 8, 10). In this regard, virtually no  $\Delta^6$ 16:0-ACP desaturase activity was detected in the 100,000  $\times$  g supernatant of *T. alata* endosperm homogenates when assays were conducted in the absence of ferredoxin or molecular oxygen (Fig. 4).  $\Delta^6$ 16:0-ACP desaturase activity was also reduced when catalase was omitted from assays. Furthermore, the inclusion of 1 mM KCN or H<sub>2</sub>O<sub>2</sub> in reactions resulted in the loss of most of the desaturase activity. Such catalytic properties of the *T. alata*  $\Delta^6$ 16:0-ACP desaturase were similar to those previously described for the  $\Delta^9$ 18:0-ACP desaturase (7, 8, 10).

***Isolation of a cDNA Encoding a Diverged Acyl-ACP Desaturase from T. alata Endosperm*** - Based on functional similarities of the  $\Delta^6$ 16:0 - and  $\Delta^9$ 18:0-ACP desaturases described above, we examined whether these enzymes are also structurally related. To address this question, attempts were made to isolate a cDNA for the  $\Delta^6$ 16:0-ACP desaturase using  $\Delta^9$ 18:0-ACP desaturase-derived probes. As a first approach, a cDNA expression library prepared from poly(A)<sup>+</sup> RNA of *T. alata* endosperm was screened with antibodies against the  $\Delta^9$ 18:0-ACP desaturase of avocado (13). This method was previously used to obtain a cDNA for the  $\Delta^4$ 16:0-ACP desaturase of coriander endosperm (21). In the present study, however, antibody screening of the *T. alata* endosperm expression library yielded only cDNAs for three apparent isoforms of the  $\Delta^9$ 18:0-ACP desaturase, which were designated pTAD1, 2, and 3 (19).

As an alternative approach, PCR amplification of a  $\Delta^6$ 16:0-ACP desaturase-specific nucleotide probe was attempted using degenerate sense and antisense oligonucleotides prepared against two conserved amino acid sequences in  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases. One of the sequences (Gly-Asp-Met-Ile-Thr-Glu-Glu) is encoded by the cDNA for the  $\Delta^4$ 16:0-ACP desaturase and all known cDNAs for the  $\Delta^9$ 18:0-ACP desaturase. The second sequence (Glu-Lys-Thr-Ile-Gln-Tyr-Leu) is also encoded by the



$\Delta^4$ 16:0-ACP desaturase of cDNA and all known  $\Delta^9$ 18:0-ACP desaturase cDNAs except that of safflower (14). Products of approximately 215 base pairs obtained following one round of PCR amplification of the total *T. alata* cDNA library (in plasmid form) were screened after subcloning into the pAMP1 vector. To delineate products of the previously isolated cDNAs pTAD1, 2, and 3, colonies containing PCR-derived clones were screened in a negative manner with random-labeled probes for pTAD1, 2, and 3 and conditions of moderate to high stringency. One of the resulting clones (pEC6) that displayed weak or no hybridization to these probes encoded an amino acid sequence that was somewhat diverged from those of known  $\Delta^9$ 18:0-ACP desaturases.

When the *T. alata* endosperm library was screened with a random-labeled probe prepared from the insert of pEC6, >0.1% of the total cDNAs examined strongly hybridized to this probe. The longest of a selected portion of these cDNAs (the corresponding plasmid was designated pTAD4) contained 1279 base pairs and had an open-reading frame corresponding to a 387-amino-acid polypeptide with considerable identity to known  $\Delta^4$ 16:0- and  $\Delta^9$ 18:0-ACP desaturases (Fig. 5). Based on similarity of flanking bases to the consensus sequence proposed by Lütcke et al. (34), the translational start site of the cDNA insert of pTAD4 likely occurs at nucleotide 17. In addition, from homology with  $\Delta^4$ 16:0- and  $\Delta^9$ 18:0-ACP desaturases, the mature peptide encoded by pTAD4 likely begins at amino acid 33. As such, the 32 amino acids preceding this residue correspond to a putative plastid transit peptide as is present in all acyl-ACP desaturases characterized to date.

Interestingly, the cDNA insert of pTAD4 lacks nucleotide sequence for 6-7 amino acids found near the amino terminus of all previously characterized  $\Delta^9$ 18:0-ACP desaturases. This region is also altered in the cDNA for the coriander  $\Delta^4$ 16:0-ACP desaturase (21) as compared to cDNAs for  $\Delta^9$ 18:0-ACP desaturases. In this case, the coding sequence for 15 amino acids is absent in the  $\Delta^4$ 16:0-ACP desaturase cDNA relative to the castor  $\Delta^9$ 18:0-ACP desaturase cDNA (13) (Fig. 5). The pTAD4-encoded peptide also contains 2 less amino acids at its carboxyl terminus than both the  $\Delta^4$ 16:0- and  $\Delta^9$ 18:0-

ACP desaturases. Despite these differences, the interior regions of the putative desaturase encoded by pTAD4 share significant identity with portions of the primary structures of  $\Delta^4$ 16:0- and  $\Delta^9$ 18:0-ACP desaturases, and the spacing between conserved regions of amino acids is the same in all three desaturase types. Overall, the mature peptide encoded by the cDNA insert of pTAD4 shares 66% identity with the castor  $\Delta^9$ 18:0-ACP desaturase and 57% identity with the coriander  $\Delta^4$ 16:0-ACP desaturase, disregarding any missing amino acids.

**Activity of an *E. coli*-expressed cDNA for a Diverged Acyl-ACP Desaturase of *T. alata* Endosperm** - To determine the activity of the desaturase corresponding to the cDNA insert of pTAD4, the mature peptide-encoding region of this clone was expressed in *E. coli* with expression driven by the T7 RNA polymerase promoter of the vector pET3d (Novagen). When assayed with [ $1\text{-}^{14}\text{C}$ ]16:0-ACP, crude extracts of isopropyl-1-thio- $\beta$ -D-galactopyranoside-induced recombinant *E. coli* catalyzed the synthesis of [ $^{14}\text{C}$ ]16:1 (Fig. 6). In addition, the methyl ester of the 16:1 product displayed mobility on argentation TLC plates similar to that of a methyl petroselinic acid (18:1 $\Delta^6$ ) standard, suggesting that this monounsaturated product is a  $\Delta^6$  isomer. Detectable acyl-ACP desaturase activity was absent in extracts of *E. coli* harboring the pET3d vector without cDNA insert or in uninduced recombinant *E. coli*. Furthermore, like the activity found in *T. alata* endosperm extracts, the desaturase expressed in *E. coli* displayed an *in vitro* substrate preference for 16:0-ACP and exhibited no detectable activity in the absence of reduced ferredoxin (Fig. 7).

The [ $^{14}\text{C}$ ]16:0 moiety produced *in vitro* from the *E. coli*-expressed desaturase was conclusively identified as a  $\Delta^6$  isomer through gas chromatography-mass spectrometry analysis of its dimethyl disulfide derivative (Fig. 8). In the mass spectrum shown, the ions 145, 177, 187, and 364  $m/z$  are diagnostic for a [ $1\text{-}^{14}\text{C}$ ]16:1 $\Delta^6$  moiety. Significant amounts of non-radiolabeled or [ $^{12}\text{C}$ ]16:1 $\Delta^6$  were also detected among the desaturase assay products. This was indicated by the presence of the additional ions 143, 175, and 362  $m/z$  in the mass spectrum of [ $1\text{-}^{14}\text{C}$ ]16:1 $\Delta^6$  as well as by an

enrichment in the abundance of ions 187  $m/z$  (Fig. 8). It is unlikely that the non-radiolabeled 16:1 $\Delta^6$  resulted from *in vivo* synthesis in *E. coli*. In this regard, *E. coli* does not normally produce 16:1 $\Delta^6$  (35). Furthermore, gas chromatographic analysis of fatty acids of *E. coli* expressing the pTAD4-encoded desaturase failed to detect any 16:1 $\Delta^6$  in the bacterial lipids (data now shown). Given the relatively high concentrations of *E. coli* protein used in these assays, unlabeled 16:1 $\Delta^6$  likely arose from the *in vitro* desaturation of endogenous *E. coli* 16:0-ACP present in crude bacterial extracts.

Of note, expression levels of the *T. alata* cDNA in *E. coli* appeared to be low relative to that often obtained with DNA inserts placed behind the T7 RNA polymerase promoter (36). The expressed protein, for example, could not be distinguished on Coomassie-stained SDS-polyacrylamide gels of either the total soluble or insoluble protein fractions of lysed *E. coli* (data not shown). Also suggestive of low expression levels in *E. coli*, the specific activity of  $\Delta^6$ 16:0-ACP desaturase in recombinant *E. coli* extracts (Fig. 7) was typically half of that detected in *T. alata* endosperm homogenates (Table I).

The results presented here demonstrate the involvement of a novel soluble  $\Delta^6$ 16:0-ACP desaturase in the synthesis of  $\Delta^6$  hexadecenoic acid in the endosperm of *T. alata*. The activity of this enzyme has several properties similar to those previously described for the  $\Delta^9$ 18:0-ACP desaturase. These include the requirement of reduced ferredoxin for detectable *in vitro* activity, the stimulation of activity by catalase, and the inhibition of activity by potassium cyanide and hydrogen peroxide. The existence of a  $\Delta^6$ 16:0-ACP desaturase in *T. alata* endosperm was confirmed by the isolation of a cDNA for this enzyme. While the amino acid sequence deduced from this cDNA shares some identity with  $\Delta^9$ 18:0- and  $\Delta^4$ 16:0-ACP desaturases, these findings, together with those previously obtained for petroselinic acid biosynthesis (19, 21), indicate that natural variations in the primary structures of acyl-ACP desaturases can give rise to novel enzymes with altered substrate recognition and double bond positioning properties.

The major difference between the primary structures of the mature  $\Delta^6$ 16:0-,  $\Delta^4$ 16:0-, and  $\Delta^9$ 18:0-ACP desaturases occurs in a region near their

amino termini. In this region, the *T. alata*  $\Delta^6$ 16:0-ACP desaturase contains 6 less amino acids than the castor  $\Delta^9$ 18:0-ACP desaturase. Similarly, this portion of the coriander  $\Delta^4$ 16:0-ACP desaturase lacks 15 amino acids relative to the castor  $\Delta^9$ 18:0-ACP desaturase. Without intending to be limited to any particular theory, one possibility is that differences in recognition of substrate chain length (16:0-ACP *versus* 18:0-ACP) and/or double bond positioning of these desaturases are associated with this divergence in the primary structures of these enzymes. Alternatively, this region of the amino terminus of  $\Delta^9$ 18:0-ACP desaturase may not contribute significantly to the catalytic properties of this enzyme. As such, while again not intending to be limited to any particular theory, if the  $\Delta^4$  and  $\Delta^6$ 16:0-ACP desaturases evolved from the  $\Delta^9$ 18:0-ACP desaturase, then there may have been little selective pressure to maintain this region intact in the variant 16:0-ACP desaturases.

Ultimately, an understanding of how differences in the amino acid sequences of  $\Delta^4$ 16:0-,  $\Delta^6$ 16:0-, and  $\Delta^9$ 18:0-ACP desaturases contribute to variations in their functional properties will require comparisons of the three-dimensional structures of these enzymes. In this regard, elucidation of the crystal structure of the castor  $\Delta^9$ 18:0-ACP desaturase is currently in progress (37). With such information, it will be possible to overlap amino acid sequences of the  $\Delta^4$  and  $\Delta^6$ 16:0-ACP desaturases onto the three-dimensional structure of  $\Delta^9$ 18:0-ACP desaturase to more precisely identify residues associated with the different substrate recognition and double bond positioning properties of these enzymes. This could eventually lead to the design of "tailor-made" desaturases that are capable of inserting double bonds into a variety of positions of acyl moieties of a range of carbon chain lengths.

An interesting observation from the studies described above was the lack of detectable amounts of 16:1 $\Delta^6$  in lipids of *E. coli* expressing the *T. alata* cDNA. Similarly, Thompson et al. (14) reported that expression of the safflower  $\Delta^9$ 18:0-ACP desaturase cDNA did not lead to the *in vivo* production of oleic acid in recombinant *E. coli*. The latter result can be explained by the fact that *E. coli* contains little 18:0-ACP (38). However, 16:0-ACP is a major

component of the acyl-ACP pool of *E. coli*. Therefore it is unlikely that the lack of 16:1 $\Delta^6$  synthesis in *E. coli* expressing the *T. alata* cDNA is due to the presence of insufficient substrate for the desaturase. In addition, *E. coli* has been reported to contain ferredoxin (39), the apparent electron donor for the  $\Delta^6$ 16:0-ACP desaturase. However, as proposed by Thompson et al. (14), *E. coli* ferredoxin may not functionally interact with plant acyl-ACP desaturases. Alternatively, *E. coli* may not have adequate amounts of ferredoxin in a reduced form as required for  $\Delta^6$ 16:0-ACP desaturase activity.

In addition to 16:1 $\Delta^6$ , *T. alata* seed contains the unusual fatty acid 18:1 $\Delta^8$ , which composes about 2% weight of the oil of this tissue (23). We have previously shown that petroselinic acid (18:1 $\Delta^6$ ) is formed by elongation of 16:1 $\Delta^4$ -ACP in Umbelliferae endosperm (22). In an analogous manner, we predict that 18:1 $\Delta^8$  arises from the elongation of 16:1 $\Delta^6$ -ACP rather than from the  $\Delta^8$  desaturation of 18:0-ACP. Unlike the synthesis of petroselinic acid, though, elongation of 16:1-ACP in *T. alata* endosperm is likely not a major pathway as the ratio of amounts of 16:1 $\Delta^6$ :18:1 $\Delta^8$  in this tissue is approximately 40:1. In contrast, the ratio of amounts of 16:1 $\Delta^4$ :18:1 $\Delta^6$  in endosperm of the Umbelliferae coriander is more than 1:500 (22, 25).

Finally, significant efforts have been directed toward the development of transgenic crops that produce high value specialty oils (4, 40, 41). Using methodologies currently well known in the art, transgenic plants could be produced (42, 43) which would contain and express the  $\Delta^6$ 16:0-ACP desaturase gene and which would produce high levels of 16:1 $\Delta^6$ . In this regard, oils rich in 16:1 $\Delta^6$  may have properties suitable for industrial use. Like petroselinic acid, 16:1 $\Delta^6$  can be oxidatively cleaved at its double bond to yield adipic acid, a precursor of nylon 6,6. In addition, high palmitic acid (16:0) mutants of crop plants including soybean (44) and *Brassica campestris* (45) are available that could serve as appropriate backgrounds for transgenic expression of the cDNA for the *T. alata*  $\Delta^6$ 16:0-ACP desaturase. Still the success of such research would likely require additional studies to determine whether enzymes other than  $\Delta^6$ 16:0-ACP desaturase are

specialized for the synthesis and metabolism of 16:1 $\Delta^6$  in *T. alata* endosperm. For example, a petroselinoyl-ACP-specific thioesterase has been identified in Umbelliferae endosperm extracts that efficiently releases petroselinic acid from ACP and, as a result, makes this fatty acid available for subsequent storage in triacylglycerol (46). A related enzyme may also be required for high levels of 16:1 $\Delta^6$  accumulation in transgenic plants.

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**What Is Claimed Is:**

1. An isolated  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
2. A transgenic plant cell comprising heterologous DNA encoding a  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
3. The transgenic plant cell of Claim 2 wherein said heterologous DNA is as shown in SEQ. I.D. NO.: 1.
4. The transgenic plant cell of Claim 2 wherein said  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase is expressed and active.
5. The transgenic plant cell of Claim 2 as a seed or a propagate of the seed.
6. An isolated DNA encoding a  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
7. The DNA of Claim 6 wherein said DNA encodes a *Thunbergia alata*  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
8. The DNA of Claim 6 wherein said DNA is as shown in SEQ. ID NO.: 1.
9. A mRNA derived from a DNA as shown in SEQ. I.D. NO.: 1.
10. A transgenic plant comprising heterologous DNA encoding a  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
11. The transgenic plant of Claim 10 wherein said heterologous DNA is as shown in SEQ. I.D. NO.: 1.
12. The transgenic plant of Claim 10 wherein said  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase is expressed and active.
13. A vector comprising DNA encoding a  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase.
14. A method for modifying the fatty acid content of a plant seed, the method comprising transforming the plant which produces the seed with a heterologous DNA encoding a  $\Delta^6$  - palmitoyl-acyl carrier protein desaturase, and introducing a double bond at the sixth carbon atom from the carboxyl end of a 16 carbon saturated fatty acid in the plant seed through the activity of the expressed desaturase.
15. The method of Claim 14 wherein the double bond at the sixth carbon atom from the carboxyl end of the 16 carbon saturated fatty acid is

introduced by a  $\Delta^5$  - palmitoyl-acyl carrier protein desaturase from *Thunbergia alata*.

16. The method of Claim 14 further comprising oxidative cleavage of the monounsaturated fatty acid formed by the method of Claim 14.
17. A plant seed having a modified fatty acid content derived through the process of Claim 14.
18. A plant seed having a modified fatty acid content derived through the process of Claim 15.
19. A plant seed having a modified fatty acid content derived through the process of Claim 16.

1/8

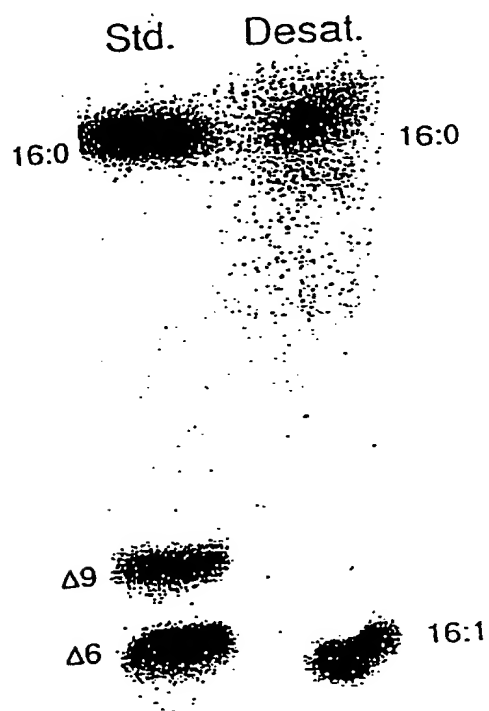


FIGURE 1

2/8



FIGURE 2

3/8

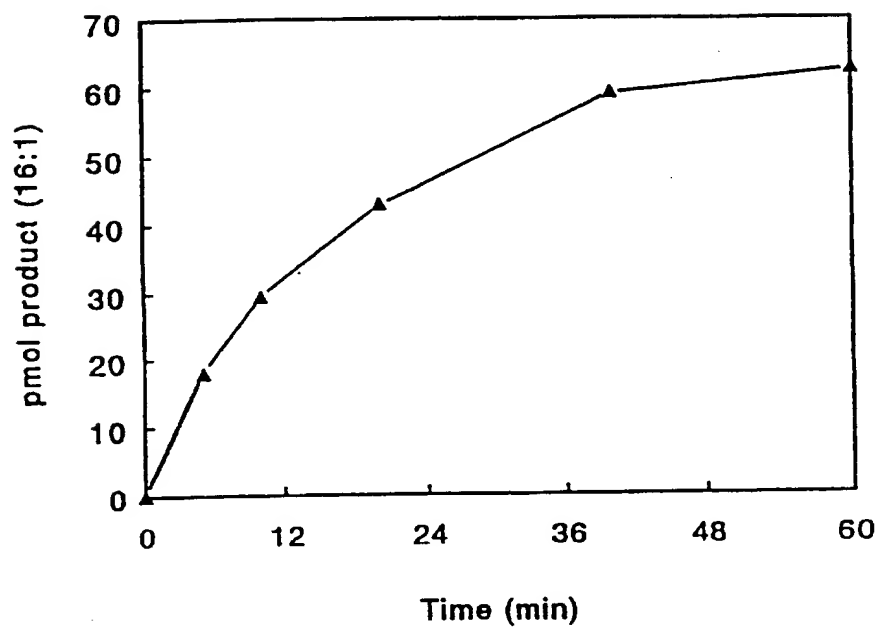


FIGURE 3

4/8

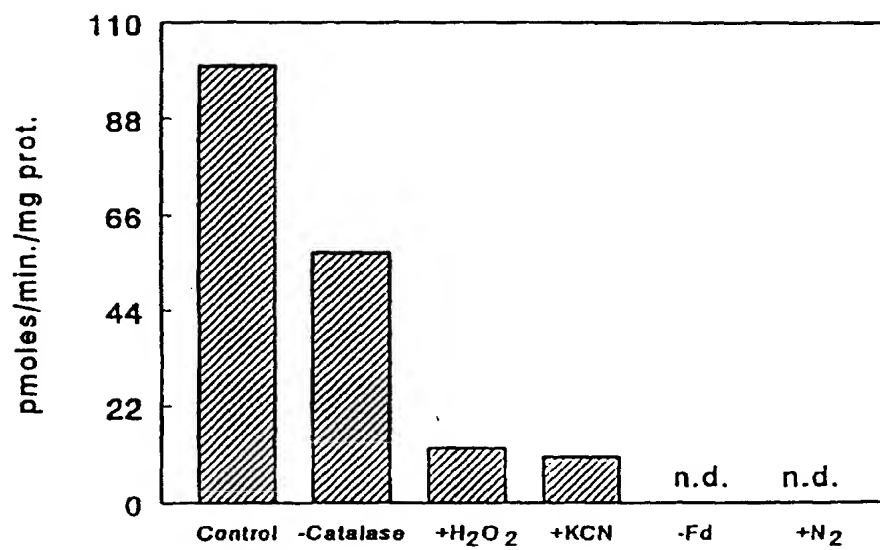


FIGURE 4

5/8

46 ATTTTGTAAAGTGAAGATGGCATTGGTATTCAAGAGTATAGGAGCCCATAGACTCTCTTGTACTTTAAATTAGCTTCACCAGCTTTGTACCAC 97  
 46 M A L V F K S I G A N K T P P C T L N L A S P A L Y H 27  
 49 M A L K L N P F L S Q T Q K L P S F A L P P M A S T R S 28  
 44 M A N K L N A L M T L Q C P K R N N F T R I A P P Q A G R V R 31

46 ACCAGAGTCACAATGGCTTCGACTATTACTCACCTCCGCCACTCAAAGAT.....AGAAAAATATCGTCTACTCGACGA...GTAAGG..... 178  
 46 T R V T M A S T I T H P P P L K D - - R K I S S T R R - V R - - - 54  
 49 P K F Y : : : : L K S G S K E V E N L K : P F M P P : E : H V Q V 61  
 44 S K V S : : : : L N A S : L V F : K L K A G R P - - - E : - - - 57

46 ACATATCCGTTGGCTCCAGAGAAGGCTGAAATCTTCAATTCTATGCACGGGTGGGTTGAAGACACCAATCTCCCTTTCCTGAAGCCGGTGGAGGAGTCG 277  
 46 T Y P L A P E K A E I F N S M N G W V E D T I L P F L K P V E E S 87  
 49 : H S M P : Q : I : : : K : L D N : A : E M : : V H : : : : K C 94  
 44 - - - - - - - - - - L : : : L E : : A R : M : : V H : : S : : M : 82

46 TGCAGCCGACGGACTTCTCCGGACTCCACTTCTGATGGGTTCACGAGCAAGTGGAGAGCTTCGTAACGGAACGGCCGATCTCCCTGATGATTAC 376  
 46 W Q P T D F L P D S T S D T S D F H E Q V E E L R K R T A D L P D D Y 120  
 49 : : : Q : : : : : P A : : : : D : : : R : : : E : A K E I : : : : 127  
 44 : : : Q : Y : : : P : : : A : E D : : : K : M : E : A K : I : : : E : 115

46 TTAGTTCGATTGGTGGGCAATGGTGCAGGAGGAAGCCCTTCCGACGTATCAACAATGCTTAACGACAGATGTGATATACGATGAGAGCGGGCCC 475  
 46 L V A L V G A M V T E E A L P T Y Q T M L N T T D V I Y D E S G A 153  
 49 F : V : : : D : I : : : : : : : : : : : : : L : G V R : : T : : 160  
 44 F : V : : : D : I : : : : : : : : : : : : : M S : : : R C : G : K : D T : : 148

46 AGCCCTGTGCTTGGGCGGTTTGGACCCGGGCTTGGACCCCTGAAGAGAACAGCATGGTGTATTTGTCAACAAGTATCTCTATCTTCCGGTCTGTGC 574  
 46 S P V P W A V V T R A V T A E E N R H G D I V N K Y L Y L S G R V 186  
 49 : : T S : : I : : : : : : : : : : : : : L L : : : : : : : : 193  
 44 Q : T S : : I : : : : : : : : : : : : : L L : : : : : : : : 181

46 CATATGAAGCAATTCAGAGACTATTCAATACTTGATTGGCTCGGCGATGGATCTGGTGGGACAAACCCGTACCTAGCATATATCTACACGTCG 673  
 46 D M K Q I E K T I O Y L I G S G M D P G A D N M P Y L A Y I Y T S 219  
 49 : : R : : : : : : : : : : : : : : : : : R T E : S : : : G F : : : : 226  
 44 : : R M : : : : : : : : : : : : : : : : : T K T E : C : : : N G F : : : : 214

46 TATCAGGAGAGGGCTACAGCGATCTCCCATGGAAGTCTGGCCGGCTAGCGAGGAGAGGAGAGATGAACTGGCTCAGATTGTGGAACAATTTCT 772  
 46 Y Q E R A T A I S H G S L G R L A R Q K G E M K L A Q I C G T I S 252  
 49 F : : : : : F : : : : : N T A : Q : K E N : D I : : : : : : : : A 259  
 44 F : : : : : F : : : : : A N T A K : : Q N Y : D K N : : : V : : N : A 247

46 GCCGATGAGAAGCGGCAGGCGGGCTACTCGAAATCGTGAGAGCACTATTCCAGTTCGATCCAGAAGGCACAATGTTGGCGTTGGCATACATGATG 871  
 46 A D E K R M E A A Y S K I V E K L F E L D P E G T M L A L A Y M H 285  
 49 : : : R : : : : : T : : : : : : : : : : : I : : : D : : V : : F : D : : 292  
 44 S : : : : : A T : : : T : : : : : : : : : : A : I : : D T : V I : F S D : : 280

46 AAGATGAAGATTGTAATGCCAGCTCGTCTGATGCACGATGGGAAGGATCCGGACATGTTCAACATTTCTCTGCTGTGTCCGAGCGACTGGGGATTAC 970  
 46 K M K I V M P A R L M H D G K D P D H F O H F S A V S Q R L G I Y 318  
 49 R K : : S : : : : N : : Y : : R : D N L : D : : : : : A : : : : V : 325  
 44 R K : : Q : : : A N A : Y : : S : D M L : K : : T : : : : Q I : : V : 313

46 ACTGCAAGGAGTATACGGACATCTGGAGCATATGATAGCGCGGTGGGAGTGGATAAGCTGACGGGGCTGAGCGGGGAGGGCCGAGGGCGCAGGAT 1069  
 46 T A K E Y T D I L E H N I A R V G V D K L T G L S G E G R R A Q D 351  
 49 : : : D : A : : : : F L V G : K : : : : : : : : : A : : Q K : : : 358  
 44 S : M D : C : : : : D F L V D K : M : A : M : : : : : : : : : K : : E 346

46 TACGTGTGGGGTTGCCGATGAGGTTAGGAAGGTGGAGGAGAGGGCCGAGCGGTGGGCGGAGATATATCGCAT...GTTCTTTTACGTGGATCTTT 1165  
 46 Y V C G L P M R F R K V E E R A Q A M A E N I S H - V P F S W I F 383  
 49 : : : R : : : : P : I : : : : : : : : : : : G R : K E A P T - N : : : : : 390  
 44 : : : S : A A K I : R : : : K V : G K E K K A V L P V A : : : : : 379

46 GGGAGAAGAGTG.....TAGTCTCAGTCTCAGTCTCACTCGGTCACTGTGTTGTTGTTCTATGATCAAGAAATAAGTGCAATGCCACCCTTATTCTC 1258  
 46 G R R V - - - 387  
 49 D : Q : K L - 396  
 44 N : Q I I I - 385

FIGURE 5



6/8

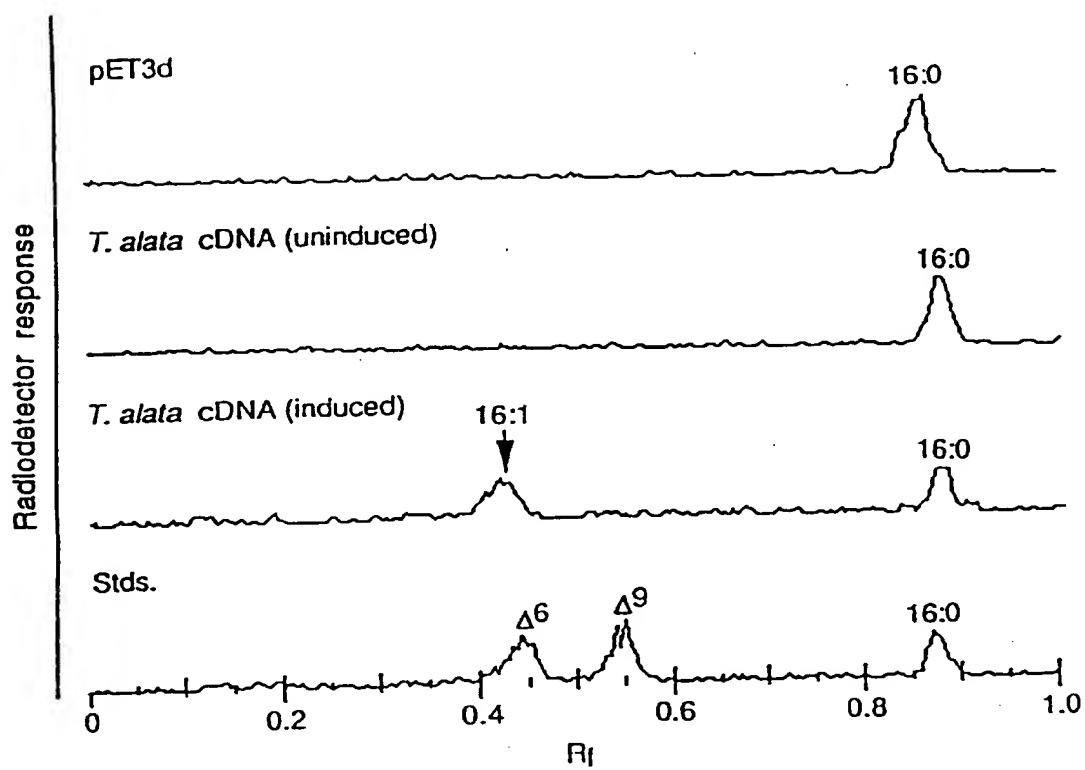


FIGURE 6

7/8

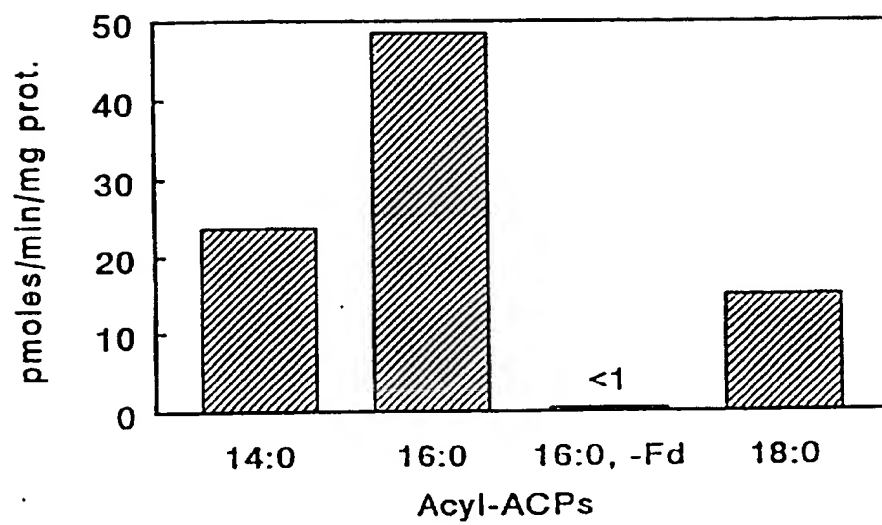


FIGURE 7

8/8

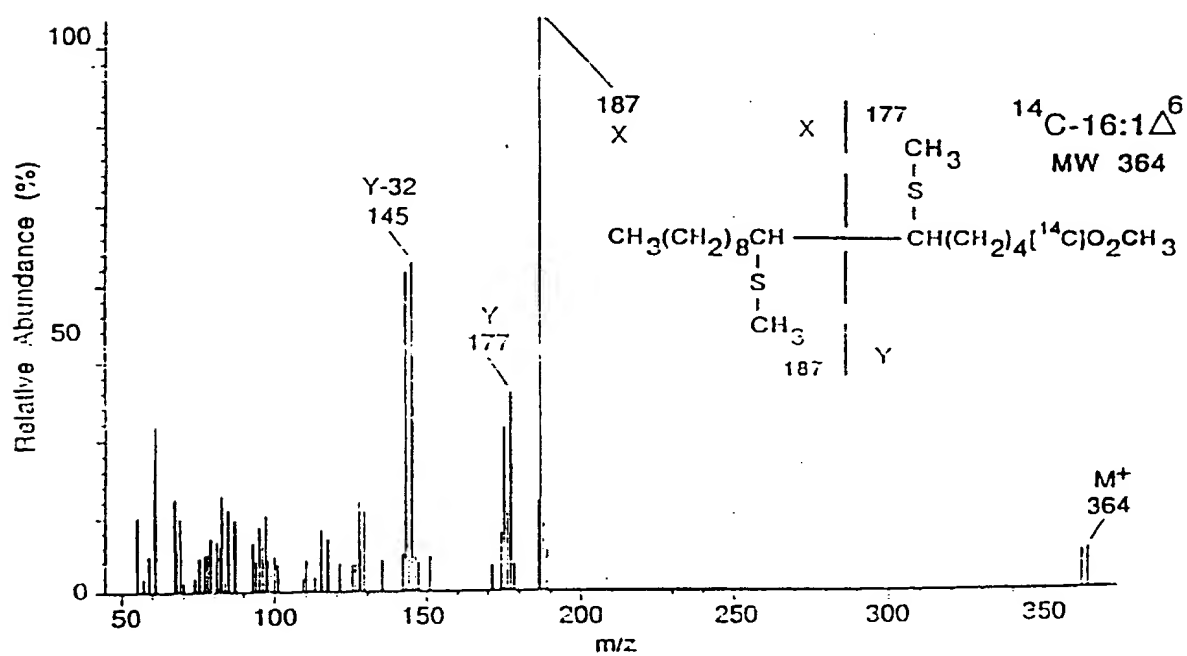


FIGURE 8